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Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

98303872.0

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LABELLED GLUTAMINE AND LYSINE ANALOGUES

The present invention relates to a class of compounds useful in the diagnosis of sites of thrombosis, embolism or infection, pharmaceutical formulations containing them, their use in the diagnosis of disease and methods for their preparation.

Prior approaches to thrombus imaging radiopharmaceuticals include radiolabelled fibrinogen or plasminogen; radiolabelled fragment E_1 of human fibrin; radiolabelled plasminogen activators such as tissue plasminogen activator (t-PA) and labelled anti-fibrin antibodies. Methods based on the detection of sites of platelet accumulation such as the administration of radiolabelled platelets (e.g. using ^{111}In oxine) or radiolabelled anti-platelet antibodies have also been described. More recent efforts have focused on radiolabelled peptides or polypeptides such as the cell adhesion motif RGD (where R, G and D are the standard abbreviations for the amino acids arginine, glycine and aspartic acid respectively); platelet factor 4 or fragments thereof or anticoagulant peptides such as disintegrins.

Factor XIII is a plasma glycoprotein which is present in blood and certain tissues in a catalytically inactive (or zymogen) form. Factor XIII is transformed into its active form Factor XIIIa by thrombin in the presence of calcium ions. Factor XIIIa is also known as plasma transglutaminase, fibrinoligase or fibrin-stabilising factor. The final step in the formation of a blood clot is the covalent crosslinking of the fibrin which is formed by the proteolytic cleavage of fibrinogen by thrombin. Fibrin molecules align and the enzyme Factor XIIIa catalyses covalent crosslinking of the NH_2 and $CONH_2$ groups of lysyl and glutaminyl residues respectively giving structural rigidity to the blood clot. The crosslinking stabilises the fibrin clot structure and confers resistance to fibrinolysis. The crosslink formation is

an important facet of normal blood coagulation and wound healing as well as pathological conditions such as thrombosis. It may also be implicated in atherosclerosis and tumour growth and metastasis. WO 91/16931 discloses that radiolabelled analogues of Factor XIII (in which the active site has been inactivated by amino acid substitution) are useful as thrombus imaging radiopharmaceuticals.

Factor XIIIa is also known to catalyse the incorporation of low molecular weight amines into the γ-glutamine sites of proteins. Similarly Factor XIIIa also catalyses the incorporation of low molecular weight glutamine analogues into lysyl residues. Thus such low molecular weight amines (or glutamine analogues) function as competitive inhibitors of the Factor XIIIa-induced lysyl/glutaminyl crosslinking of proteins.
A range of synthetic amines have been described which are competitive inhibitors of the uptake of labelled putrescine (1,4-butanediamine) into N,N'-dimethylcasein catalysed by pig liver transglutaminase [L.Lorand *et al.*, Biochem., 18, 1756(1979)].

WO 89/00051 (Cytrx Biopool Ltd.) claims a method for targeting fibrin deposits using a labelled compound which is covalently bound to fibrin by Factor XIIIa. The fibrin binding compound is stated to be "any peptide that is a substrate for the blood enzyme commonly known as Factor XIIIa". Preferred peptides are said to include the tetrapeptide sequence -Asn-Gln-Glu-Gln- (or NQEQ in standard amino acid abbreviation notation). Also disclosed is the 12-mer peptide sequence from the NH_2 terminus of the alpha-2 antiplasmin enzyme:
NH_2-Asn-Gln-Glu-Gln-Val-Ser-Pro-Leu-Thr-Leu-Thr-Leu-Leu-Lys-OH
together with a synthetic analogue:
NH_2-Asn-Gln-Glu-Gln-Val-Ser-Pro-Tyr-Thr-Leu-Thr-Leu-Leu-Lys-OH,
(denoted NQEQVSPLTLTLLK and NQEQVSPYTLTLLK respectively). The latter was radiolabelled with ^{125}I and shown to be taken up in thrombin clots *in vitro*.

It has now been discovered that synthetic analogues of lysine and glutamine labelled with suitable detectable moiety can also function as substrates for the enzyme Factor XIIIa. The use of suitable protecting groups provides compounds which are less susceptible to *in vivo* metabolism especially by peptidases, and are hence more useful targeting agents.

The present invention provides the following compounds:

$$Y\text{-}(CR_2)_n\text{-}X\text{-}NHJ$$

where:

X is C=O or CR_2;

n is an integer of value 1 to 6;

Y is $L(A)_m$- or R^1R^2CR- where L is a metal complexing agent, A is -CR_2- , -CR=CR- , -C≡C- , -NRCO- , -CONR- , -SO_2NR- , -$NRSO_2$- , -CR_2OCR_2- , -CR_2SCR_2- , -CR_2NRCR_2- , a C_{4-8} cycloheteroalkylene group, a C_{4-8} cycloalkylene group, a C_{5-12} arylene group, a C_{3-12} heteroarylene group or a polyalkyleneglycol, polylactic acid or polyglycolic acid moiety;

m is an integer of value 0 to 10;

where one of R^1 and R^2 is -$NH(B)_pZ^1$ and the other is -$CO(B)_qZ^2$ where

p and q are integers of value 0 to 30, and

each B is independently chosen from Q or an amino acid,

where Q is a cyclic peptide;

Z^1 and Z^2 are protecting groups;

J and each R group are independently chosen from H, C_{1-4} alkyl, C_{1-4} alkenyl, C_{1-4} alkynyl, C_{1-4} alkoxyalkyl or C_{1-4} hydroxyalkyl;

with the provisos that:

(i) the total number of amino acid residues in the R^1 and R^2 groups does not exceed 30;

(ii) when X is CR_2, then Y is -CRR^1R^2 and Z^2 is a metal complexing agent;

(iii) when Y is $-CRR^1R^2$ then at least one of R^1 and R^2 bears at least one detectable moiety.

The invention also includes kits for the preparation of the above compounds labelled with a detectable moiety, and the use of these and related compounds in the diagnosis or therapy of thrombosis, embolism, atherosclerosis, inflammation or cancer.

By the term "cyclic peptide" is meant a sequence of 5 to 15 amino acids in which the two terminal amino acids are bonded together by a covalent bond which may be a peptide or disulphide bond or a synthetic non-peptide bond such as a thioether, phosphodiester, disiloxane or urethane bond.

By the term "amino acid" is meant an *L*- or *D*-amino acid, amino acid analogue or amino acid mimetic which may be naturally occurring or of purely synthetic origin, and may be optically pure, i.e. a single enantiomer and hence chiral, or a mixture of enantiomers. Preferably the amino acids of the present invention are optically pure. By the term "amino acid mimetic" is meant synthetic analogues of naturally occurring amino acids which are isosteres, i.e. have been designed to mimic the steric and electronic structure of the natural compound. Such isosteres are well known to those skilled in the art and include but are not limited to depsipeptides, retro-inverso peptides, thioamides, cycloalkanes or 1,5-disubstituted tetrazoles [see M. Goodman, Biopolymers, 24, 137, (1985)].

By the term "protecting group" is meant a biocompatible group which inhibits or suppresses *in vivo* metabolism of the peptide or amino acid at the amino or carboxyl terminus. Such groups are well known to those skilled in the art and are suitably chosen from, for the amine terminus (Z^1): acetyl, Boc (where Boc is *tert*-butyloxycarbonyl), Fmoc (where Fmoc is fluorenylmethoxycarbonyl), benzyloxycarbonyl, trifluoroacetyl, allyloxycarbonyl, Dde [i.e. 1-(4,4-dimethyl-2,6-

dioxocyclohexylidene)ethyl], Npys (i.e. 3-nitro-2-pyridine sulfenyl), or a metal complexing group; and for the carboxyl terminus (Z^2): a carboxamide, *tert*-butyl ester, benzyl ester, cyclohexyl ester, amino alcohol or a metal complexing group. Preferably the protecting group is a metal complexing group, most preferably it is a metal complexing group bound to a metal i.e. a metal complex. The carboxyl terminus of peptides is particularly susceptible to *in vivo* cleavage by carboxypeptidase enzymes. Consequently, the metal complexing group or metal complex is preferably attached at the carboxyl terminus. When either R^1 is $-NH(B)_{p-1}QZ^1$ or R^2 is $-CO(B)_{q-1}QZ^2$ then the protecting group may be the covalent bond which closes the cyclic peptide (Q) ring.

A "detectable moiety" is a moiety which emits a signal or suitable for diagnostic imaging of the human body and may be a radioisotope for radiopharmaceutical imaging or therapy, a paramagnetic metal or species for MRI contrast imaging, a radiopaque group or metal for X-ray contrast imaging, a gas microbubble ultrasound contrast agent or a suitable dye for detection by external light imaging. Preferably, the imaging moiety is a metal ion, most preferably it is a radiometal.

When Y is $-CRR^1R^2$ and $-(CR_2)$-X-NHJ is $-(CH_2)_4NH_2$ (i.e. the amino acid side chain of lysine) then preferably either one or both of R^1 and R^2 comprises one or more peptide fragments of α_2-antiplasmin, fibronectin or beta-casein, such peptide fragments comprising at lest three and preferably 4 – 20 amino acid residues. Most preferably, R^1 and R^2 include at least one of these fragments whenever Y is $-CRR^1R^2$. The amino acid sequences of α_2-antiplasmin, fibronectin, beta-casein, fibrinogen and thrombospondin can be found in the following references: α_2-antiplasmin precursor [M.Tone *et al.*, J.Biochem, 102, 1033, (1987)]; beta-casein [L.Hansson *et al,* Gene, 139, 193, (1994)]; fibronectin [A.Gutman *et al*, FEBS Lett., 207, 145, (1996)]; thrombospondin-1 precursor [V.Dixit *et al*, Proc. Natl. Acad. Sci., USA, 83, 5449, (1986)];

R.F.Doolittle, Ann. Rev. Biochem., 53, 195, (1984).

Preferably the amino acid sequence is taken from the N-terminus of α_2-antiplasmin, i.e.:

NH_2-Asn-Gln-Glu-Gln-Val-Ser-Pro-Leu-Thr-Leu-Thr-Leu-Leu-Lys-OH

or variants of this in which one or more amino acids have been exchanged, added or removed such as:

NH_2-Asn-Gln-Glu-Gln-Val-Ser-Pro-Leu-Thr-Leu-Thr-Leu-Leu-Lys-Gly-OH,

NH_2-Asn-Gln-Glu-Ala-Val-Ser-Pro-Leu-Thr-Leu-Thr-Leu-Leu-Lys-Gly-OH,

NH_2-Asn-Gln-Glu-Gln-Val-Gly-OH.

When the compound of the present invention is a peptide, i.e. Y is R^1R^2CR- the number of amino acid residues is preferably 2 to 30, most preferably 3 to 20, especially 3 to 15.

Preferred compounds have J equal H, i.e. terminate in an NH_2 group. X is preferably C=O, i.e. compounds of formula Y-$(CR_2)_n$-$CONH_2$ are preferred. Most preferred compounds are of formula Y-$(CR_2)_x$-$(CH_2)_2CONH_2$ or Y-$(CR_2)_y$-$(CH_2)_4NH_2$ where x is an integer of value 0 to 4, and y is an integer of value 0 to 3. Compounds having the same side chain as glutamine, i.e. glutamine analogues of formula Y-$(CR_2)_x$-$(CH_2)_2CONH_2$ are especially preferred.

Suitable non-metallic radioisotopes for use in the present invention include but are not limited to: radioiodine such as ^{123}I, ^{125}I, ^{131}I, preferably ^{123}I; positron emitters such as ^{18}F, ^{11}C or ^{75}Br and isotopes for therapy e.g. ^{211}At.

The compounds of this invention which comprise metal complexing agents preferably have only a single type of targeting molecule attached, i.e. the -$(CR_2)_n$-X-NHJ substituent. Other substituents on the complexing agent may be present, but the -$(CR_2)_n$-X-NHJ substituent is the one which is expected to be primarily responsible for the biolocalisation properties. Metal complexes of the present invention may contain one or more metal ions which may be the same or different. Thus in some

circumstances polynuclear complexes may have advantageous properties such as certain metal clusters which have superparamagnetic properties and are hence particularly useful as MRI contrast agents. Preferred metal complexes of the present invention involve only a single metal ion. When the metal of the metal complex is a radiometal, it can be either a positron emitter (such as ^{68}Ga or ^{64}Cu) or a γ-emitter such as ^{99m}Tc, ^{111}In, ^{113m}In or ^{67}Ga. Suitable metal ions for use in MRI are paramagnetic metal ions such as gadolinium(III) or manganese(II). Most preferred radiometals for diagnostic imaging are γ-emitters, especially ^{99m}Tc. Metal complexes of certain radionuclides may be useful as radiopharmaceuticals for the radiotherapy of various diseases such as cancer or the treatment of thrombosis or restenosis. Useful radioisotopes for such radiotherapeutic applications include: ^{90}Y, ^{89}Sr, ^{67}Cu, ^{186}Re, ^{188}Re, ^{169}Er, ^{153}Sm and ^{198}Au. Whichever metal complex is chosen, it is strongly preferred that it is bound to the Factor XIIIa substrate in such a way that it does not undergo facile metabolism in blood with the result that the metal complex is cleaved from the Factor XIIIa substrate before the labelled Factor XIIIa substrate reaches the desired *in vivo* site to be imaged. The Factor XIIIa substrate is therefore preferably covalently bound to the metal complexes of the present invention.

These metal ions are complexed using a metal complexing agent, or more preferably a chelating agent. The chelating agents comprise 2-10 metal donor atoms covalently linked together by a non-coordinating backbone. Preferred chelating agents have 4-8 metal donor atoms and have the metal donor atoms in either an open chain or macrocyclic arrangement or combinations thereof. Most preferred chelating agents have 4-6 metal donor atoms and form 5- or 6-membered chelate rings when coordinated to the metal centre. Such polydentate and/or macrocyclic chelating agents form stable metal complexes which can survive challenge by endogenous competing ligands for the metal *in*

vivo such as transferrin or plasma proteins. Alternatively, it is possible to use monodentate complexing agents that form stable complexes with the desired metal ion even though they do not form chelate rings upon metal coordination. Examples of known complexing agents of this kind, which are particularly suitable for use with ^{99m}Tc, are hydrazines, phosphines, arsines, or isonitriles.

Examples of suitable chelating agents are bidentate such as diamines or diphosphines, tridentate such as monoaminedithiols, or tetradentate such as diaminedioximes (US 4615876) or such ligands incorporating amide donors (WO 94/08949); the tetradentate ligands of WO 94/22816 ; N_2S_2 diaminedithiols, diamidedithiols or amideaminedithiols; N_3S thioltriamides; N_4 ligands such as tetraamines, macrocyclic amine or amide ligands such as cyclam, oxocyclam (which forms a neutral technetium complex) or dioxocyclam; or dithiosemicarbazones. The above described ligands are particularly suitable for technetium, but are useful for other metals also. Other suitable ligands are described in Sandoz WO 91/01144, which includes ligands which are particularly suitable for indium, yttrium and gadolinium, especially macrocyclic aminocarboxylate and aminophosphonic acid ligands. Ligands which form non-ionic (i.e. neutral) metal complexes of gadolinium are known and are described in US 4885363. The ligand may also comprise a short sequence of amino acids such as the Cys/amino acid/Cys tripeptide of WO 92/13572 or the peptide ligands described in EP 0719790 A2.

It is well known to prepare chelating agents which have attached thereto a functional group ("bifunctional chelates"). Functional groups which have been attached to chelating agents include: amine, carboxylic acid, cyanate, thiocyanate, maleimide and active ester such as N-hydroxysuccinimide. Examples of chelate-amine conjugates for diaminedioxime ligands are given in WO 95/19187. When the desired

Factor XIIIa substrate functionality is an amine, the ligands of the present invention can be prepared by reaction of a bifunctional compound which contains both an amine group (preferably protected by use of suitable protecting groups known to those skilled in the art), and a reactive group such as a sulphonyl chloride, acid chloride, active ester or an alkyl/benzyl halide. The reactive group can then be coupled to either the pendant amine group of a bifunctional chelate, or used to derivatise one or more of the amine donor atoms of a N-containing ligand. Alternatively, a mono-protected diamine could be reacted with a bifunctional chelate with a pendant active ester or carboxyl group to give the protected amine group linked to the ligand system *via* an amide bond. In both synthetic routes outlined above, the resulting ligand-protected amine conjugate is then deprotected under suitable conditions to give the desired amine-functionalised ligand. When the desired Factor XIIIa substrate functionality is a carboxamide group, the desired ligands can be prepared e.g. by reaction of a omega-haloalkyl carboxamide of suitable chain length with a bifunctional chelate with a pendant amine group, giving the desired carboxamide-linked ligand.

The metal complexes of the present invention may be prepared by reacting a solution of the metal in the appropriate oxidation state with the ligand at the appropriate pH. The solution may preferably contain a ligand which complexes weakly to the metal (such as chloride, gluconate or citrate) i.e. the metal complex is prepared by ligand exchange or transchelation. Such conditions are useful to suppress undesirable side reactions such as hydrolysis of the metal ion. When the metal ion is ^{99m}Tc, the usual starting material is sodium pertechnetate from a ^{99}Mo generator. Technetium is present in ^{99m}Tc-pertechnetate in the Tc(VII) oxidation state, which is relatively unreactive. The preparation of technetium complexes of lower oxidation state Tc(I) to Tc(V) therefore usually requires the addition of a suitable reducing agent such as stannous ion to facilitate

complexation. Further suitable reductants are described below.

The metal complex should also preferably be of low lipophilicity (since high lipophilicity is often related to non-specific uptake), and exhibit low plasma protein binding (PPB) since plasma-bound label again contributes to undesirable high, non-specific blood background for the imaging agent.

Thus the present invention relates mainly to diagnostic agents for imaging sites in the mammalian body where the enzyme Factor XIIIa is up-regulated and fibrin is deposited. The present agents are particularly useful for the diagnostic imaging of the human body. The agents comprise substrates for the enzyme Factor XIIIa which are labelled with a metal complex suitable for external imaging such as a radiometal (for scintigraphy) or a paramagnetic metal ion (for MRI). The metal complex of the present invention has a pendant amino or carboxamide functional group which is available for covalent linking to protein glutamyl carboxamide or lysyl amine groups respectively by the enzyme Factor XIIIa. The intimate relationship of fibrin and Factor XIIIa highlights the potential use of the agents of the present invention for the diagnosis of disease states where there is both fibrin deposition or accumulation and up-regulation of Factor XIIIa. Increased fibrin deposition is known to be characteristic of diseases such as thrombosis, atherosclerosis, fibrotic liver, and disseminated intravascular coagulation. Fibrin is also deposited at sites of tissue inflammation associated with many disease processes, such as infection, autoimmune disease or cancer. Factor XIIIa and tissue transglutaminase are up regulated during known physiological conditions. During apoptosis and generation of new matrix protein structures elevated levels of the enzymes are seen. The present agents may thus also be used for the detection of apoptosis and diseases states such as arthritis where increased matrix protein deposition occurs. Since Factor XIIIa is up-regulated at the site of interest *in vivo* (i.e. thrombus, embolism etc.) this

provides a localisation mechanism for the metal complexes of the present invention. The covalently linked metal complexes can then be imaged externally by radionuclide scintigraphy or magnetic resonance imaging (MRI) hence providing a non-invasive means of diagnosing the disease site.

So far as the therapeutic aspects of this invention are concerned, the inventors have preliminary *in vivo* data (not reported in detail herein) indicating that clots produced in the presence of labelled peptides of the present invention (as per Example 9 below) are smaller than those produced in the absence of the labelled peptide. On this basis it is proposed that the peptides herein defined, typically containing 4 – 30 e.g. about 10 amino acid residues, are effective as drugs for increasing the rate of clot lysis e.g. by acting as potent inhibitors of fibrin cross linking in clots.

The present invention also relates to kits for the preparation of metal complexes linked to Factor XIIIa substrates. The kits are designed to give sterile products suitable for human administration, e.g. *via* injection into the bloodstream. Possible embodiments are discussed below. When the detectable moiety is ^{99m}Tc, the kit would comprise a vial containing the free ligand or chelating agent for the metal together with a pharmaceutically acceptable reducing agent such as sodium dithionite, sodium bisulphite, ascorbic acid, formamidine sulphinic acid, stannous ion, Fe(II) or Cu(I), preferably a stannous salt such as stannous chloride or stannous tartrate. Alternatively, the kit could contain a metal complex which, upon addition of the radiometal or paramagnetic metal, undergoes transmetallation (i.e. ligand exchange) giving the desired product. For ^{99m}Tc, the kit is preferably lyophilised and is designed to be reconstituted with sterile ^{99m}Tc-pertechnetate (TcO_4^-) from a ^{99m}Tc radioisotope generator to give a solution suitable for human administration without further manipulation.

The agents of the present invention may also be provided in a unit dose form ready for human injection and could for example be supplied in a pre-filled sterile syringe. When the detectable moiety is a radioactive isotope such as ^{99m}Tc, the syringe containing the unit dose would also be supplied within a syringe shield (to protect the operator from potential radioactive dose).

The above kits or pre-filled syringes may optionally contain further ingredients such as buffers; pharmaceutically acceptable solubilisers (e.g. cyclodextrins or surfactants such as Pluronic, Tween or phospholipids); pharmaceutically acceptable stabilisers or antioxidants (such as ascorbic acid, gentisic acid or *para*-aminobenzoic acid) or bulking agents for lyophilisation (such as sodium chloride or mannitol).

The following examples illustrate the preparation of compounds of the present invention and their use in imaging. The syntheses of particular compounds of the present invention are given in Examples 1-2, and their radiolabelling with ^{123}I or ^{99m}Tc in Examples 3 and 4. Evidence for increased plasma stability *in vitro* is given in Example 5. Evidence for uptake in blood clots *in vitro* and *in vivo* is given in Examples 7 and 9 respectively, with normal rat biodistribution of the radiolabelled compounds reported in Example 8.

The *in vitro* plasma stability of ^{123}I-Compound 1 is poor (see Example 5), presumably due to protease activity. The introduction of protecting groups at both the carboxy and amino termini as for radiolabelled Compounds 2-8 confers a substantial increase in plasma stability.

Compounds 2, 3, 4, 6 and 8 is all exhibit high *in vitro* clot uptake and hence avidity for the clot. The other compounds are of less potency with compound 5 showing a significant reduction in uptake. This strongly suggests that the Gln-2 (i.e. glutamine residue in the 2-position) is an essential amino acid residue in the sequence.

Details of the biodistribution in normal rats and in the fresh and aged clot models are given in Examples 8 and 9. The blood clearance rate of these compounds is relatively fast with biological half lives between 1-2 hours. The biodistribution of ^{99m}Tc-Compound 3 is given as a representative example, in this case the $t_{½}$ of 2h is estimated. The rapid clearance from background tissues such as blood, lung, heart and muscle shows that the agent of the present invention possess favourable pharmacokinetics for imaging and shows their potential as radiodiagnostics.

Although some hepatobiliary excretion is seen for these compounds, the main route of excretion is *via* the urinary tract.

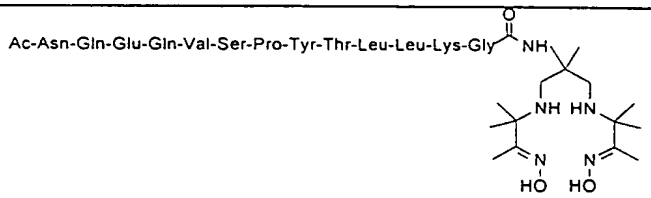
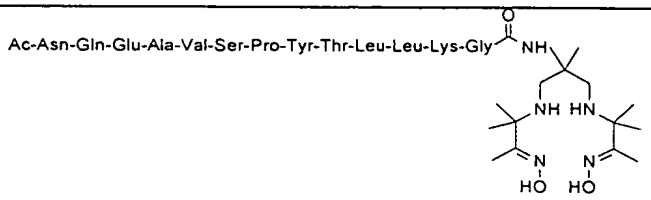
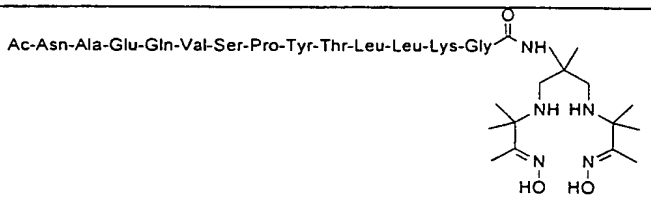
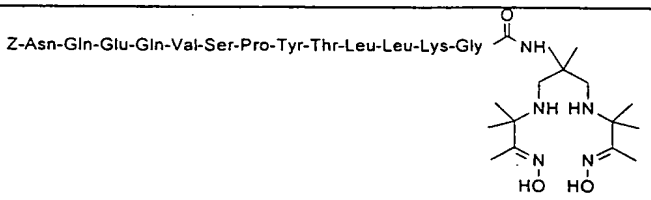
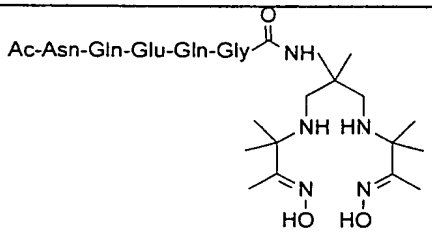
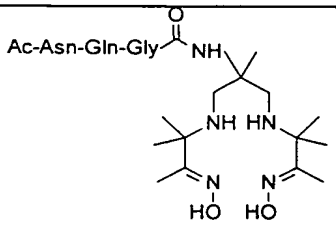
Uptake into fresh and aged clots in the rat models for ^{99m}Tc-Compounds 2-4 is very good (relative concentration or RC= 5-15), with clot to background tissue ratios very favourable for imaging (>5).

^{99m}Tc-Compounds 2-8 have improved plasma stability compared with ^{99m}Tc-Compound 1 (RC=1.5), which may be responsible for the improved *in vivo* clot uptake seen with these compounds. For ^{99m}Tc-Compound 5 the clot uptake is reduced (RC=1.2) suggesting that Gln-2 is essential for uptake.

Comparison of the clot uptake results of Example 9 for fresh and aged clots, shows that the present agents exhibit uptake which is constant and independent of the age of the clot. Thus such agents will have improved imaging capability for pre-existing clots, such as those found with pulmonary embolism.

EXPERIMENTAL

In the following table, Z is benzyloxycarbonyl.

Peptide	Compound
NH_2-Asn-Gln-Glu-Gln-Val-Ser-Pro-Tyr-Thr-Leu-Leu-Lys-OH	1
Ac- Asn-Gln-Glu-Gln-Val-Ser-Pro-Tyr-Thr-Leu-Leu-Lys-Gly-NH_2	2
	3
	4
	5
	6
	7
	8

Example 1: Syntheses of Compounds 1 and 2

The protected peptide Ac-Asn(Trt)-Gln(Trt)-Glu(OtBu)-Gln(Trt)-Val-Ser(tBu)-Pro-Tyr(tBu)-Thr(tBu)-Leu-Leu-Lys(Boc)-Gly-OH was assembled on a 2-chlorotrityl resin by anchoring Fmoc-Lys(Boc) to the resin, and then successive deprotections/coupling cycles with the appropriate protected amino acids (as described in P. Lloyd-Williams, F. Albericio and E. Girald; *Chemical Approaches to the Synthesis of Peptides and Proteins*, CRC Press, 1997). The title compound was obtained by cleavage using 0.1% TFA in dichloromethane, deprotection and purification by RP-HPLC (System A).

Mass Spec. Analysis (FAB)

Compound 1	Theoretical molecular weight:	1419.6
	Experimental molecular weight [M+H]+	1419.7
Compound 2	Theoretical molecular weight:	1516.7
	Experimental molecular weight [M+H]+	1517.7

Example 2: Syntheses of Compounds 3-8

The appropriate protected peptide was assembled as in Example 1 with the appropriate protected amino acids and then coupled with 6-aminomethyl-3,3,6,9,9-pentamethyl-4,8-diazaundecane-2,10-dione dioxime (prepared as described in WO 95/19187). The title compounds were obtained by 0.1% TFA in dichloromethane cleavage, deprotection and purified by RP-HPLC (System A).

Mass Spec. Analysis (ES+)

Compound 3:	Theoretical molecular weight:	1816.1
	Experimental molecular weight [M+H]+	1816.2
Compound 4:	Theoretical molecular weight:	1759.1
	Experimental molecular weight [M+H]+	1759.0
Compound 5:	Theoretical molecular weight:	1759.1
	Experimental molecular weight [M+H]+	1759.0

Compound 6:	Theoretical molecular weight:	1908.2
	Experimental molecular weight [M+H]+	1908.1
Compound 7:	Theoretical molecular weight:	1013.2
	Experimental molecular weight [M+H]+	1013.0
Compound 8:	Theoretical molecular weight:	656.8
	Experimental molecular weight [M+H]+	656.6

Example 3: I-123 labelling of Compound 1 and Compound 2

Ammonium acetate buffer (200μl, 0.2M, pH 4.0) was added to the ligand solution (20μl, 20μg), and $Na^{127}I$ (10μl, 1.5μg) in an Eppendorf tube. The solution was mixed thoroughly and $Na^{123}I$ (5-50μl, 111MBq) was then added. The solution was mixed thoroughly prior to addition of PAA solution (10μl, 0.01M), further mixing followed. The activity of the preparation was measured. In all cases the required product was separated from reaction by-products and unlabelled substrates by HPLC.

Example 4: Tc-99m labelling of Compounds 3-8

A 0.1ml aliquot of the compound dissolved in H_2O (1mg/ml) was transferred to a nitrogen-filled 10ml glass vial together with deoxygenated saline (0.9% w/v, 1ml) and 0.035ml aqueous NaOH (0.1M). To this solution was added technetium generator eluate (1ml, approx. 0.4GBq) and then aqueous stannous chloride solution (0.1ml, ca.10μg). The labelling pH was 9.0-10.0. Vials were incubated at ambient laboratory temperature (15-25°C) for 30 minutes to effect labelling. The resulting preparation was either diluted to the desired radioactive concentration or HPLC purification was performed (System B) to remove unlabelled starting material and radioactive impurities prior to testing. After purification the organic solvent was removed under vacuum and the sample was redissolved in about 5ml 0.1M phosphate buffer pH 7.4 to give a working concentration of 6-9MBq/ml. Radiochemical purity was assessed before

use by the thin layer chromatography (TLC) system described below:

i) ITLC SG 2cm x 20cm eluted with 0.9% w/v saline

ii) Whatman No.1 2cm x 20cm eluted with 50 : 50 v/v acetonitrile : H_2O

The labelled substrates remain at, or close to, the origin in TLC system (i) and move close to the solvent front in system (ii). When analysed by appropriate detection equipment the radiochemical purity is typically in excess of 85% labelled compound.

Example 5: *In vitro* plasma stability of Compounds 1-8

To a portion of compound (50μl, 10MBq/ml) was added an equal volume of plasma (rat or human) or saline. The mixtures were incubated at 37°C and the stability measured by HPLC (system C) at 0, 30 and 120 minutes. The saline dilution acted as a control.

Compound	Species	% intact at 120 min.
^{123}I-Cmpd 1	Human	0
	Rat	nd
^{123}I-Cmpd 2	Human	98
	Rat	99
^{99m}Tc-Cmpd 3	Human	95
	Rat	97
^{99m}Tc-Cmpd 4	Human	94
	Rat	97
^{99m}Tc-Cmpd 5	Human	91
	Rat	88
^{99m}Tc-Cmpd 6	Human	nd
	Rat	80
^{99m}Tc-Cmpd 7	Human	100
	Rat	nd
^{99m}Tc-Cmpd 8	Human	97
	Rat	100

nd - not done

Example 6: HPLC Systems

Flow Rate: 1ml/min in all systems.

System A

Column Waters C18 250x4.5mm. Particle size 4 microns

Gradient: Elution Profile 10-60%B in 25 min.

Eluent A: 0.1% aqueous TFA

Eluent B: 0.1% TFA in acetonitrile

System B

Column Waters C18 150x3.9mm. Particle size 4 microns

Gradient: Elution Profile 0-100%B in 22 min.

Eluent A: 0.1% aqueous TFA

Eluent B: 0.1% TFA in acetonitrile

System C

Column Waters C18 150x3.9mm. Particle size 4 microns

Gradient: Elution Profile 0-100%B in 20 min.

Eluent A: 50mM NH_4OAc buffer (pH 5.6)

Eluent B: acetonitrile

Example 7: Incorporation into human plasma clots

Incorporation of radiolabelled substrates into fibrin was investigated by induction of an *in vitro* human plasma clot in the following manner. To a siliconised 5 ml glass vial was added, (a) 800µl of *Tris*(hydroxymethyl)aminomethane buffered saline pH 7.5 containing calcium chloride (50mM Tris, 150mM sodium chloride, 4mM calcium chloride.), (b) about 40µl of physiological salt solution containing 100 units of thrombin per ml, (c) about 400µl of human plasma containing the radiolabelled substrate at a concentration of typically 10kBq/ml. To aid

induction of clot a roughened glass rod was added to the reaction vial. Control vials were prepared similarly but with the omission of thrombin and calcium chloride.

After incubation of the test solution at ambient laboratory temperature (ca. 20°C) for 60 minutes the reaction was discontinued with the addition of about 400µl of a cold solution of 33.5mM ethylenediaminetetra-acetic acid disodium salt. Clots were separated from serum by vacuum filtration onto 0.45 µM nitrocellulose filters (pre-soaked in 1.5% BSA/*tris*(hydroxymethyl)aminoethane buffered saline pH 7.5 containing 0.1% Tween 20) and washed with about 2 x 10ml of *tris*(hydroxymethyl)aminomethane buffered saline pH 7.5 containing Tween 20 to a final concentration of 0.1%v/v. The proportion of total radioactivity was calculated by counting in suitable detection apparatus.

The fraction of radioactivity retained on the filter, after subtraction of the non-specific binding determined from the control, is a measure of incorporation into the filtered clots.

compound	% retained (with thrombin)	% retained (no thrombin)	% specific uptake*
^{123}I-Cmpd 1	14.6	2.0	12.6
^{123}I-Cmpd 2	38.6	0.2	38.4
^{99m}Tc-Cmpd 3	39.3	0.3	39.0
^{99m}Tc-Cmpd 4	34.8	0.3	34.5
^{99m}Tc-Cmpd 5	3.3	0.5	2.8
^{99m}Tc-Cmpd 6	41.3	0.7	40.6
^{99m}Tc-Cmpd 7	12.5	0.1	12.4
^{99m}Tc-Cmpd 8	25.9	1.4	24.5

* % retained in plasma clot assay (with thrombin) - % retained in plasma clot assay (no thrombin)

Example 8: Normal rat biodistribution

The resolution of a clot image is dependant on the combination of rate of incorporation of the radiopharmaceutical and its blood/tissues clearance rate. For this reason the biodistribution of several compounds has been determined in rats. Male Wistar (100-150g) rats were injected i.v. with 0.1-0.2 ml of radiolabelled tracer solution (8MBq/ml) and dissected at different times post-injection. The %ID in each of the selected tissues was measured. Some animals were kept in metabolism cages to be able to determine the %ID excreted in urine and faeces. The dissection times used for the agent were 15, 30, 60, 240 min. Data are shown as %ID, Mean±, (n=3).

^{99m}Tc-Compound 3

	15min	30 min	60 min	240 min
Muscle	14.7	10.4	5.1	2.9
Blood	5.3	1.8	1.6	0.6
Kidney	7.6	4.9	4.6	3.4
Urine	14.9	34.3	37.0	42.0
Lung	0.7	0.4	0.3	0.3
Liver	6.2	4.1	4.0	3.0
GI Tract	13.5	15.1	18.0	20.7
Heart	0.2	0.1	0.1	0.04

Example 9: Incorporation into clots induced in a rat model

Rat Inferior vena cava model (IVC)

The rats (Male Wistar,250-350g) were anaesthetised with 15% urethane. After laparotomy, the vena cava was isolated and freed of surrounding fat tissue. A platinum wire (1.5cm x 0.5mm) was inserted into

the inferior vena cava and 5 min post surgery 0.4ml of ellagic acid (1.2×10^{-4} M) was injected intravenously through the femoral vein previously canulated, and the clot was allowed to form. The average weight of the clots formed in this model was around 27mg, n=32, (5-50mg range). The compounds were injected 5 min (fresh clot) and 60 min (aged clot) post-induction. After 60 min the animals were sacrificed and the clot removed, weighed and counted. Other tissues e.g. blood, lung, heart, were also dissected and counted . The uptake of tracer into the clot was determined as the relative concentration (cpm/g of clot by dose/g animal) and clot to background tissue.

Results:

Fresh Clots

	^{123}I-Cpd 1	^{123}I-Cpd 2	^{99m}Tc-Cpd 3	^{99m}Tc-Cpd 4	^{99m}Tc-Cpd 5
%ID/g	0.5±0.13	4.95±0.8	1.35±0.4	6.0±2.6	0.54±0.1
Rel.Conc.	1.63±0.4	14.5±2.2	5.1±1.2	15.9±6.1	1.2±0.2
Clot/Blood	1	7	10	6.5	1.6
Clot/Lung	nd	10	8	10	1.5
Clot/Heart	nd	19	17	21	2.8
Clot/Liver	nd	21	6	6	0.8

Rel. Conc.(RC) = $\frac{\text{\%id/g of clot}}{\text{\%id/g in rest of body}}$

Aged Clots

	^{123}I-Cpd 2	^{99m}Tc-Cpd 3	^{99m}Tc-Cpd 4	^{99m}Tc-Cpd 5
%ID/g	5.44±1.7	2.32±0.6	4.1±1.18	0.57±0.3
Rel.Conc.	14.4±3.8	5.3±1.8	11±3.3	1.3±0.8
Clot/Blood	8	15	14	1.4
Clot/Lung	12	9	15	1.3
Clot/Heart	23	18	30	2.4
Clot/Liver	20	4	6	0.7

Abbreviations

Ac	Acetyl
Boc	tert-butyloxycarbonyl
ES	electrospray
FAB	fast atom bombardment
Fmoc	fluorenylmethoxycarbonyl
HPLC	high performance liquid chromatography
RCP	radiochemical purity
RP-HPLC	reverse phase high performance liquid chromatography
TFA	trifluoroacetic acid
TLC	thin layer chromatography

CLAIMS

1. A compound of formula:

$$Y\text{-}(CR_2)_n\text{-}X\text{-}NHJ$$

where:

X is C=O or CR_2;

n is an integer of value 1 to 6;

Y is $L(A)_m$- or R^1R^2CR- where L is a metal complexing agent,

A is $-CR_2-$, -CR=CR- , -C≡C- , -NRCO- , -CONR- , $-SO_2NR-$, $-NRSO_2-$, $-CR_2OCR_2-$, $-CR_2SCR_2-$, $-CR_2NRCR_2-$, a C_{4-8} cycloheteroalkylene group, a C_{4-8} cycloalkylene group, a C_{5-12} arylene group, a C_{3-12} heteroarylene group or a polyalkyleneglycol, polylactic acid or polyglycolic acid moiety;

m is an integer of value 0 to 10;

where one of R^1 and R^2 is $-NH(B)_pZ^1$ and the other is $-CO(B)_qZ^2$ where

p and q are integers of value 0 to 30, and

each B is independently chosen from Q or an amino acid residue,

where Q is a cyclic peptide;

Z^1 and Z^2 are protecting groups;

J and each R group are independently chosen from H, C_{1-4} alkyl, C_{1-4} alkenyl, C_{1-4} alkynyl, C_{1-4} alkoxyalkyl or C_{1-4} hydroxyalkyl;

with the provisos that:

(i) the total number of amino acid residues in the R^1 and R^2 groups does not exceed 30;

(ii) when X is CR_2, then Y is $-CRR^1R^2$ and Z^2 is a metal complexing agent;

(iii) when Y is $-CRR^1R^2$ then at least one of R^1 and R^2 bears at least one detectable moiety.

2. The compound of claim 1 where R^1 or R^2 includes one or more peptide fragments of α_2-antiplasmin, fibronectin or beta-casein, said peptide fragment containing at least three amino acid residues.

3. The compound of claim 1 or claim 22 where J is H.

4. The compound of claim 3 of formula:

$$Y\text{-}(CR_2)_x\text{-}(CH_2)_2CONH_2 \text{ or } Y\text{-}(CR_2)_y\text{-}(CH_2)_4NH_2$$

where x is an integer of value 0 to 4, and y is an integer of value 0 to 3.

5. The compound of any one of claims 1 to 4 where Y is $-CRR^1R^2$.

6. The compound of claims any one of 1 to 5 where at least one of Z^1 and Z^2 is a metal complexing agent.

7. The compound of claim 6 where Z^2 is a metal complexing agent and Z^1 is not a metal complexing agent.

8. A metal complex of the compounds of claim 6 or claim 7.

9. The metal complex of claim 8 where the metal is a radiometal.

10. The radiometal complex of claim 9 where the radiometal is ^{99m}Tc.

11. A preparation for human administration comprising the compound of any one of claims 1 to 10.

12. A kit comprising the compound of any one of claims 1 to 7 useful in the preparation of the metal complexes of any one of claims 8 to 10.

13. Use for the diagnosis of sites of thrombosis or embolism of a compound of formula:

$$Y\text{-}(CR_2)_n\text{-}X\text{-}NHJ$$

where:

X is C=O or CR_2;

n is an integer of value 1 to 6;

Y is $L(A)_m$- or R^1R^2CR- where L is a metal complexing agent,

A is $-CR_2$- , -CR=CR- , -C≡C- , -NRCO- , -CONR- , $-SO_2NR$- , $-NRSO_2$- , $-CR_2OCR_2$- , $-CR_2SCR_2$- , $-CR_2NRCR_2$- , a C_{4-8} cycloheteroalkylene group, a C_{4-8} cycloalkylene group, a C_{5-12} arylene group, a C_{3-12} heteroarylene group or a polyalkyleneglycol, polylactic acid or polyglycolic acid moiety;

m is an integer of value 0 to 10;

where one of R^1 and R^2 is $-NH(B)_pZ^1$ and the other is $-CO(B)_qZ^2$ where

p and q are integers of value 0 to 30, and

each B is independently chosen from Q or an amino acid residue,

where Q is a cyclic peptide;

Z^1 and Z^2 are protecting groups;

J and each R group are independently chosen from H, C_{1-4} alkyl, C_{1-4} alkenyl, C_{1-4} alkynyl, C_{1-4} alkoxyalkyl or C_{1-4} hydroxyalkyl;

with the provisos that:

(i) the total number of amino acid residues in the R^1 and R^2 groups does not exceed 30;

(ii) when X is CR_2, then Y is $-CRR^1R^2$;

(iii) when Y is $-CRR^1R^2$ then at least one of R^1 and R^2 bears at least one detectable moiety.

14. Use for the diagnosis of sites of thrombosis or embolism of a radiometal complex of the compound defined in claim 13, wherein at least one of Z^1 and Z^2 is a metal complexing agent.

ABSTRACT

LABELLED GLUTAMINE AND LYSINE ANALOGUES

Synthetic analogues of lysine and glutamine are provided which function as substrates for the fibrin-stabilising enzyme Factor XIIIa even when labelled with a detectable moiety. The use of suitable protecting groups provides compounds which possess reduced susceptibility to *in vivo* metabolism especially by peptidases, and are hence useful agents for the diagnosis of thrombosis, embolism, atherosclerosis, inflammation or cancer.